UNIVERSITY OF ALBERTA

POTENTIAL OF GLYCOMACROPEPTIDE AND β-LACTOGLOBULIN: ISOLATION AND CHARACTERIZATION

BY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of Master of Science

IN

FOOD SCIENCE AND TECHNOLOGY

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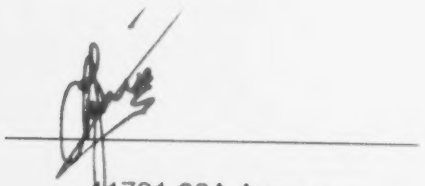

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ABSTRACT

Different combinations of UF membranes (50,000, 30,000 and 10,000 MWCO) were examined to evaluate efficiency of separation of GMP. 30,000 MWCO UF was best suited for GMP production (50.09 % of the protein recovered was GMP). Ultrafiltration coupled with ion exchange chromatography could improve purity.

HACCP results indicated that the sanitation procedures used was adequate in preventing microbial contamination.

Glycomacropeptide did not inhibit the growth of *Pseudomonas aerugenosa* ATCC 15442, *Eschericia coli* ATCC 25922, *Listeria Innocua* ATCC 33090, *Staphylococcus aureus* MJ, *Bacillus* isolate BLDV2.

The binding of retinoic acid to β-lactoglobulin (native and charcoal delipidized) and the digestive patterns of the complex were also studied. The Kd and n values for native and charcoal treated protein were 2.1×10^{-6} M and 2.8×10^{-6} M respectively, and n values of 0.92 and 0.99, respectively.

A 70% decrease in hydrolysis by trypsin was observed when retinoic acid was to bound charcoal treated or native protein.

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TABLE OF CONTENTS

CHAPTER	PAGE
1. INTRODUCTION	1
1.1. Milk proteins	2
1.1.1. Caseins	2
1.1.2. Whey proteins	3
1.2. Milk derived functional proteins/peptides	4
1.2.1. Proteins	4
1.2.2. Bioactive peptides derived from milk proteins	5
1.3. Glycomacropeptide	6
1.4. β-lactoglobulin	8
1.5. Research objectives	10
1.6. References	16
2. GLYCOMACROPEPTIDE	24
2.1. Introduction	24
2.2. Materials and methods	26
2.2.1. Chemicals	26
2.2.2. Preparation of glycomacropeptide (GMP) from sodium caseinate	26
2.2.3. Ultrafiltration (UF)	27
2.2.4. High performance liquid chromatography (HPLC) evaluation	28
2.2.5. Amino acid analysis	28
2.2.6. Protein evaluation	29
2.2.7. Yield and purity determination	29
2.2.8. Hazard analysis critical control point (HACCP) evaluation	29
2.2.9. Microbial assay	31
2.3. Results and discussion	32
2.4. Conclusions	37
2.5. References	53
3. Effect of charcoal delipidization treatment of β-lactoglobulin on kinetics of β-lactoglobulin/retinoic acid complex and its tryptic hydrolysis	57
3.1. Introduction	57
3.2. Materials and methods	58
3.2.1. Chemicals	58
3.2.2. Charcoal treatment	59
3.2.3. Fluorescence spectroscopy	59
3.2.3.1. β-lactoglobulin titration	60

3.2.5. Enzymatic digestion 60
3.2.5.1. Trypsin hydrolysis 61
3.2.5.2. High Performance Liquid Chromatography (HPLC) evaluation of protein hydrolysate 61
3.3. Results and discussion 62
3.4. Conclusions 64
3.5. References 69

4. CONCLUDING CHAPTER 73
4.1. Summary of research findings 73
4.2. Recommendations for future work 75
4.2.1. Biological role of glycomacropeptide and β-lactoglobulin 76
4.2.2. Isolation process improvement 77
4.2.3. Sources of glycomacropeptide and other biologically functional peptides 79
4.3. References 80

LIST OF FIGURES

FIGURES		PAGE
2.1.	Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 1).	42
2.2.	Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 2).	43
2.3.	Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 3).	44
2.4.	Critical points of sodium caseinate hydrolysis.	45
2.5.	Critical points of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration.	46
2.6.	Growth of *Pseudomonas aerugenosa* 15442 at room temperature and tryptic soy broth supplemented with various substrates.	47
2.7.	Growth of *Eschericia coli ATCC* 25922 at room temperature and tryptic soy broth supplemented with various substrates.	48
2.8.	Growth of *Listeria Innocua* 33090 at room temperature and tryptic soy broth supplemented with various substrates.	49
2.9.	Growth of *Staphylococcus aureus MJ* at room temperature and tryptic soy broth supplemented with various substrates.	50
2.10.	Growth of *Bacillus* isolate BLDV2 at room temperature and tryptic soy broth supplemented with various substrates.	51
2.11.	Growth of *Listeria Innocua* 33090 incubated at room temperature and tryptic soy broth supplemented with 100µl of various substrates.	52
3.1.	Fluorescence titration curve of charcoal treated β-lactoglobulin variant A with retinoic acid and the kinetic parameters (Kd and n) of the protein/ligand binding	66
3.2.	Fluorescence titration curve of native β-lactoglobulin variant A with retinoic acid and the kinetic parameters (Kd and n)	67

of the protein/ligand binding

3.3. Tryptic hydrolysis profile of charcoal treated β-lactoglobulin variant A and charcoal treated β-lactoglobulin variant A/ retinoic acid complex 68

3.4. Tryptic hydrolysis profile of native β-lactoglobulin variant A and native β-lactoglobulin variant A/ retinoic acid complex 69

LIST OF TABLES

TABLE		PAGE
1.1.	Bovine milk proteins and some of their properties	13
1.2.	Biological functions of bovine milk proteins.	14
1.3.	Biological functions of bovine peptides and their origins.	15
2.1.	Protein and glycomacropeptide (GMP) content of freeze-dried retentates obtained by ultrafiltration of sodium caseinate hydrolysate.	39
2.2.	Amino acid composition of crude glycomacropeptide and purified glycomacropeptide isolated from sodium caseinate hydrolysates	40
2.3.	Microbial load of raw material, intermediate products and by-products of glycomacropeptide production from sodium caseinate solution	41

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Milk is a complex biological fluid secreted by the mammary glands of lactating female mammals. Its main purpose is to provide all the nutrients required by the newborn infants. Milk is also a source of high quality proteins, hence milk from other species has been used as food by mankind since the early days. Some examples of milk used by humans as food sources are goat's milk, sheep's milk, and cow's milk. Today, cow's milk is the most commonly used milk and is processed commercially in large quantities (1).

Milk contains numerous compounds in equilibrium with the aqueous medium either in a dispersed or solubilized form. In addition, these compounds may also

interact with each other to form complexes which renders milk its visual and functional characteristics. The composition of milk varies genetically, geographically as well as seasonally. The average composition of bovine milk is as follows: protein: 3.6%; sugar: 5.0%; fat: 3.7%; ash: 0.7% (2). The milk proteins can be broken down further into their individual components as shown in Table 1.1 (2,3).

1.1. MILK PROTEINS

Milk proteins can be divided into two main distinct types: a) caseins and, b) whey proteins. In general, 80% of total milk proteins are caseins and 15-20% are whey proteins. Historically, man has exploited the physico-chemical differences of the two proteins to manufacture different dairy products.

1.1.1. CASEINS

Caseins are random coiled structured, heat stable proteins rich in phosphorous. Their amphiphilic nature and the presence of phosphorous allows the individual casein molecules to interact with each other and with calcium phosphate to form highly hydrated spherical micellar complexes. The micelles together with fat globules impart the white or yellow-white opaque color to milk by scattering and absorbing light (1,2,3). Caseins precipitate out of milk at pH 4.6 (isoelectric point), a physico-chemical characteristic of caseins that has been utilized

in cheese manufacture and the production of different caseinate salts.

Bovine caseins can be subdivided into four families based on the homology of their amino acid sequences or primary structure (Table 1.1): α-S_1, α-S_2, β, κ caseins. α-S_2-caseins and β-caseins are rich in phosphorous (1,2,3,4). In general, α-S_1 and α-S_2 -caseins are considered to be absent from human milk and are thought to be two of the allergenic substances in milk (5,6). κ-caseins are the only glycoproteins of the casein family (8,9,10). All caseins and whey proteins can be a source of biologically functional peptides (4,7,11,12,14).

1.1.2. WHEY PROTEINS

Whey proteins consist of milk proteins that remain soluble at pH 4.6 and 20°C. Whey proteins are relatively heat labile. β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins are the four main groups of bovine proteins. The minor proteins include lactoperoxidase, lactoferrin and proteose-peptones. Depending on the source of whey (acid precipitation of caseins result in acid whey and enzymatic coagulation of caseins provide sweet cheese whey), other proteolytic breakdown of caseins fragments may also be present in varying quantities (1,2). Traditionally, whey is a waste/by-product of cheese manufacture and has been used as a source of animal feed for centuries (11). However, with the advancement of technology, whey protein concentrates (WPC) are being used extensively as ingredients in the food industry (11,12).

Milk proteins are recognized universally to be of high quality and possessing good functional characteristics. Milk proteins are used as food ingredients because of their: 1) diverse molecular structures and physico-chemical properties; 2) interactions with other food ingredients (13). In recent years, there has been an increased interest in exploiting milk proteins as a source of biological and/or functional ingredients (4,12,14). The three main areas of research in this direction are: 1) selective isolation of individual protein/peptide to produce better functional or bioactive ingredients (11,15,16); 2) selective hydrolysis and isolation of milk proteins to produce specific peptides (5,14,17,18); 3) examination of the biological activities of the proteins/peptides (14,17,18).

1.2. MILK DERIVED FUNCTIONAL PROTEINS/ PEPTIDES

1.2.1. PROTEINS

The major milk proteins, caseins, are a source of nutritional proteins but their specific bioactivities have not been fully elucidated. However, due to their interaction with calcium phosphate to form colloidal calcium phosphate micelles, they may act as a carrier of calcium and phosphate, thereby increasing the bioavailability of the two minerals (1,2,4).

Of the whey proteins, α-lactalbumin is the B subunit of the enzyme lactose synthetase which promotes the selective transfer of galactose to glucose during

lactose biosynthesis (2,3,4). The role of β-lactoglobulin is thought to be related to the binding of retinoid and hydrophobic molecules such as fatty acids because of its structural homology to plasma retinol binding protein (19,20,21). Studies on the binding parameters of retinoic acid to β-lactoglobulin had been carried out and it was shown that retinoic acid binds to β-lactoglobulin *in vitro* with a molar binding ratio of 1:1 (22). Immunoglobulins provide passive immunity function. Lactoferrin, on the other hand, promotes iron absorption due to its iron binding capability (4,11). Lactoferrin is also reported to possess antimicrobial effect (4). The biological functions of milk proteins are presented in Table 1.2 (4,11).

1.2.2. BIOACTIVE PEPTIDES DERIVED FROM MILK PROTEINS

The "white mining" of milk proteins has led to the discovery of a vast array of peptides with different biological activities. Some of the reported activities of these peptides include: opioid agonistic effect, opioid antagonisation, immunostimulation, mineral carriers, antithrombotic effect, and anti-hypertensive capability (4,7,11,12,14). A summary of the peptides and their reported biological activities are tabulated in Table 1.3.

1.3. GLYCOMACROPEPTIDES

κ-casein is a component of the casein micelles. It plays a major role in the stabilization of casein micelles in the presence of calcium ions. In fact, it is the only soluble casein component at the calcium ion level found in milk (2, 24). κ-casein is the only glycoprotein of the casein family (9,10). It contains heterogeneous quantity of carbohydrates. Some of the sugars attached include galactose, galactosamine and N-acetylneuramic acid (2,3,23). The magnitude and the sites of post-translational glycosylation are not fixed (3,23). It has been shown that the carbohydrate molecules are usually O-glycosically linked to the threonine or serine residues located between residues 127-141 of the peptide chain of κ-casein (23). The presence of genetic variants (A, B), phosphate groups and non-glycosylated κ-caseins (33-40%) only add to the heterogeneity of the protein (23,25,26). Upon heating, κ-casein forms a complex with β-lactoglobulin, thereby decreasing the chymosin or rennet hydrolytic sites of κ-casein (27,28). It is thought that this complex formation may increase cheese yields (28) because β-lactoglobulin co-precipitates with casein.

The enzymatic hydrolysis of rennet or chymosin splits κ-casein into two peptide fragments at the ^{105}Phe - ^{106} Met bond. The first peptide fragment, f1-105, is the hydrophobic para-κ-casein which co-precipitates with other casein components in the presence of calcium ions. The second peptide fragment, f106-169, is the hydrophilic C-terminal fragment called glycomacropeptide.

Glycomacropeptide remains in solution together with whey proteins after hydrolysis because it contains high amount of acidic amino acid residues (29,30). This characteristic property of κ-casein as the specific substrate of rennin is a key step in cheese production (31).

Glycomacropeptide is a hydrophilic peptide with a molecular weight of approximately 8,000 Daltons in its monomeric form (32). The monomer has a tendency to self-associate and the degree of self-association is pH dependent (33). It has been shown that between pH 3.0 and 4.5, the molecular weight of glycomacropeptide is between 30,000 and 50,000 Daltons (33).

Glycomacropeptide is heterogenous because: 1) the two genetic variants of κ-caseins, A and B, have genetic substitutions at the glycomacropeptide portion of the polypeptide chain; 2) the phosphorylation and glycosylation sites of κ-caseins are located at f106-169; 3) a diverse amount of carbohydrates attachments which can be at five different glycosylation sites; 4) 33-40% of glycomacropeptide is not glycosylated (25,34).

The roles of the sugar moieties of glycomacropeptide have not been fully elucidated though they are thought to retard the rate of chymosin hydrolysis (36). The carbohydrates are also believed to aid in the stabilization micelles in their natural environment (9,36). The sugar moieties play varying roles in the biological activities of GMP (36).

The stabilization effect of κ-casein on casein micelles can be attributed to

glycomacropeptide. The presence of numerous hydroxyamino acid residues may be the contributing factor to the micellar stabilization force. GMP contains no cysteine, arginine, histidine, phenylalanine or any other aromatic residues (37). The release of glycomacropeptide from κ-casein can be used as a tool to study the activity, kinetics of chymosin and the concentration of κ-casein in milk (29,38). The presence of glycomacropeptide in skim milk powder is also an indication of the adulteration of the product with sweet cheese whey (39,40).

Some biological functions of GMP have been reported. These include: 1) Bifidus growth promoting factor (39); 2) antigastric activity in dogs (40,41); 3) antithrombotic activity in guinea-pig (42); 4) depression of platelet aggregation (43); 5) inhibition of binding of oral actinomyces and streptococci to polystyrene surfaces (44); 6) inhibition of cholera toxin binding to its receptor (45); 7) growth inhibition of lactic acid bacteria and *Staphylococcus aureus* (46). The role of sugars in the above activities varied.

1.4. β-LACTOGLOBULIN

β-lactoglobulin is a globular protein and the major strong binding complex of whey protein (66%). To date, four genetic variants of β-lactoglobulin, (A, B, C, D) have been identified with A and B being the most common in North America. β-lactoglobulin monomer has a molecular weight of approximately 18,000. The isoelectric point of β-lactoglobulin is about 5.3 (47).

β-lactoglobulin has a tendency to self-associate and the degree of self-association is influenced by environmental conditions such as pH, temperature and ionic strength. For example, between pH 3.7 and 5.1, at 2°C, the protein occurs predominantly as an octamer (48). Above pH 7.5, the dimer dissociates into monomers (49). The degree of aggregation varies among different genetic variants with β-lactoglobulin variant A having the strongest self-association tendency (47).

The conformation and physico-chemical properties of β-lactoglobulin have been studied extensively. A comparison study of the amino acids sequences in β-lactoglobulin family showed that 33 out of the 55 amino acids residues are homologous in all members of the β-lactoglobulin family (49). These residues are instrumental to the conformational structure of the protein and provide the characteristic biological and functional properties (50). Papiz *et. al.* (19) examined the structure of β-lactoglobulin and concluded that a hydrophobic core exists within the unique β-barrel motif. This pocket is partially accessible to the surface of the protein molecule (19). It was proposed that the hydrophobic pocket serves as the binding site of β-lactoglobulin for small non-polar molecules (19). Monaco et. al. (51) proposed that the retinol binding pocket is a superficial binding pocket that is accessible to chemical or enzymatic hydrolysis (51). The β-barrel structure of β-lactoglobulin showed remarkable resemblance to plasma retinol binding protein (19,52). Therefore, 1) the conformational homology between retinol-binding protein and β-lactoglobulin (19,52); 2) the formation of strong binding complex with

retinol (49); 3) the stability of β-lactoglobulin under acidic conditions and (19); 4) the resistance of β-lactoglobulin to peptic hydrolysis (53) suggests that β-lactoglobulin may serve as a transport protein.

The interaction between ligand and the protein and the mechanism of binding depends on 1) the structure of the ligand; 2) the conformational state of the protein; 3) the presence of other substrates; 4) environmental conditions such as temperature, pH, ionic conditions. Ligand binding by proteins alters the physico-chemical properties of the protein and may protect the protein from chemical and enzymatic hydrolysis. β-lactoglobulin has a high affinity for a wide range of small organic molecules, i.e., apolar, amphipathic molecules such as fatty acids, cyclic alcohols, ketones and aldehydes (54). Because of the resistance of β-lactoglobulin to pepsin hydrolysis and its stability under very acidic conditions and the presence of vitamin A precursor receptor in the small intestine of the neonate, β-lactoglobulin has been proposed as the transport protein of this group of compounds (55). It is also suggested that the amount of ligand bound may be limited to a single aromatic ring structure because of the proposed binding site (56).

1.5. RESEARCH OBJECTIVES

Glycomacropeptide is a by-product of cheese manufacture and the extraction of glycomacropeptide from cheese whey presents a logical solution to waste disposal and the production of value-added, functional food protein.

However, upon considerable pondering with utmost consideration given to the continuous production of a series of functional food proteins and/or peptides, sodium caseinate was used as the initial raw ingredient. The use of sodium caseinate served a few purposes: 1) little denaturation would be imparted on the glycomacropeptide and the other proteins/peptides of interest; 2) the use of organic solvents and other non-food grade chemicals was avoided; 3) little sample treatment was required which decreased production time and cost; 4) potential commercial scale operation was attainable; 5) the resulting by-product after glycomacropeptide separation could be separated and collected for further hydrolysis and extraction of valuable peptides; 6) the by-product might be further processed into a functional ingredient; 7) ample amount of glycomacropeptide could be produced for functional applications.

Rennin hydrolysis coupled with ultrafiltration were chosen as the process protocol to be studied because they met the above processing qualifications. A large scale laboratory ultrafiltration unit was used to separate glycomacropeptide from other proteins/peptides. The resultant glycomacropeptide was used in a preliminary antimicrobial study.

In addition, the binding parameters of charcoal-treated β-lactoglobulin variant A to retinoic acid and the effect of tryptic digestion on the protein/ligand complex were also studied. β-lactoglobulin binds a number of small hydrophobic molecules including fatty acid and retinoic acid. Native dimeric β-lactoglobulin contains 1 mole

of fatty acids per mole of protein. It has been shown that fatty acids may compete with retinol for binding. However, it is not known whether they compete for the same binding sites. The purpose of this study was to examine the effect of charcoal stripping of bound hydrophobic molecules on β-lactoglobulin/retinoic acid binding. The study also examined the effect of trypsin digestion on β-lactoglobulin and β-lactoglobulin/retinoic acid complex over time. β-lactoglobulin has been shown to be resistant to pepsin hydrolysis and therefore may serve as a ligand carrier to increase bioavailability of fat soluble molecules.

The overall objectives of the research were 1) evaluation of production of glycomacropeptide using different molecular weight cut-off ultrafiltration membranes; 2) hazard analysis critical control plan evaluation for glycomacropeptide production from sodium caseinate; 3) preliminary evaluation of antimicrobial activity of glycomacropeptide; 4) examination of the potential of glycomacropeptide as a phenylketonuria protein supplement; 5) examination of the effect of charcoal treatment on β-lactoglobulin/retinoic acid binding; 6) effect of tryptic hydrolysis on β-lactoglobulin/retinoic acid complex over time.

Table 1.1: Bovine milk proteins and some of their properties.

Protein	Genetic variants	Molecular weights (kdal)	Isoelectric point	Composition (% total protein)
Caseins			4.6	75-85
αS_1-casein	A,B,C,D	23.6	4.44-4.76	39-46
αS_2-casein	A,B,C,D	25.2	4.2-4.6	8-11
β-casein	A_1,A_2,A_3,B, C,D,E	24	4.83-5.07	25-35
κ-casein	A,B	19	5.3-5.8	8-15
Whey proteins				15-22
β-lactoglobulin	A,B,C,D	18.3	5.13	7-12
α-lactalbumin	A,B	14.2	4.2-4.5	2-5
Serum albumin		66.3	4.7-4.9	0.7-1.3
Immunoglobulins				1.9-3.3
IgG_1		162	5.5-6.8	1.2-3.3
IgG_2		152	7.5-8.3	0.2-0.7
IgA		400		0.2-0.7
IgM		950		0.1-0.7
SC		80		0.2-0.3
Proteose-peptone		4-41		2-6

Adapted from references 1 and 2.

Table 1.2: Biological functions of bovine milk proteins.

Proteins	Biological functions
Caseins	calcium absorption
α-lactalbumin	B-subunit of lactose synthetase complex
β-lactoglobulin	retinoids and fatty acids binding
Immunoglobulins	antigen-antibody reaction
Lysozyme	anti-microbial activity
Lactoferrin	antimicrobial activity and iron binding

Adapted from references 4 and 11.

Table 1.3: Biological functions of bovine peptides and their origins.

Peptides	Origin	Functions
β-casomorphin 5	β-casein fragment 60-66	opioid agonist
Morphiceptin	β-casein fragment 60-63	
α-casein exorphin	α-S_1 casein fragment 90-96	
Serorphin	serum albumin	
α-lactorphin	α-lactalbumin fragment 50-53	
β-lactorphin	β-lactoglobulin fragment 102-105	
Casoxin 4	κ-casein fragment 35-41	opioid antagonist
Casoxin A	κ-casein fragment 58-61	
Casoxin C	κ-casein fragment 25-34	
CEI_{12}	α-S_1 casein fragment 23-34	Antihypertensive
CEI_5	α-S_1 casein fragment 23-27	peptides
CEI-C_6	α-S_1 casein fragment 194-199	
CEI-b_7	α-S_1 casein fragment 177-183	
α-S_1 fragment 194-199	α-S_1 casein fragment 194-199	immunomodulation
β-casein 63-68	β-casein fragment 63-68	immunomodulation
β-casein 191-193	β-casein fragment 191-193	
β-casomorphin-11	β-casein fragment 60-70	Calcium binding
α-S_1-caseino-phosphopeptide-9	α-S_1 casein fragment 66-74	
κ-CN 106-116	κ-casein fragment 106-116	anti-thrombotic
κ-CN 106-112	κ-casein fragment 106-112	activity

Adapted from references 4,7,11,12 and 14

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CHAPTER 2

GLYCOMACROPEPTIDE

2.1. INTRODUCTION

Milk proteins can be broken down into two main components: 1) caseins and, 2) whey proteins. During the renneting process in cheese manufacture where chymosin is utilized to produce an enzymatic or sweet curd, the κ-casein is split into two fragments at the 105-106 peptide bond consisting of phenylalanine and methionine. The peptide fragment made up of amino acids 1-105 is: 1) insoluble;

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2) contains no carbohydrate moieties; 3) has phenylalanine residues and 4) known as para-κ-casein. On the other hand, the peptide containing amino acid residues 106-169 is: 1) soluble; 2) contains heterogenous carbohydrate moieties; 3) contains no aromatic amino acids and, 4) is known as glycomacropeptide (1,2,3,4,5,6,7,8). Due to the inherent properties of the two fractions, the para κ-casein remains with the cheese curd playing a major role in stabilization of the casein micelle and the glycomacropeptide is found in the whey along with the soluble whey proteins.

Recently, there has been increased interest in selective hydrolysis and/or isolation of milk protein fractions to produce specific functional protein/peptides. Examples of the type of functionality that GMP has been associated with are: 1) antimicrobial activity; 2) inhibition of adhesion of oral bacteria to reactive surfaces such as erythrocytes; 3) an appetite suppressant in some animal species; 4) anti-thrombotic activity; and 5) bifidus growth promoting factor (9,10,11,12,13).

Large scale isolation of GMP from sweet cheese whey by ultrafiltration has been reported (14). Other isolation methods include anion exchange chromatography (15), affinity chromatography (16) and alcohol precipitation or a combination of the above (16). The objective of the this study was to investigate the feasibility of hydrolyzing sodium caseinate to produce GMP as an initial product on a large scale basis; the resultant curd was intended for extraction of other protein/peptide components, however, the other components will not be dealt with in this chapter.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Spray dried sodium caseinate powder was obtained from American Casein Company (Burlington, NJ, U.S.A.). Crystalline rennin was a product of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Whey powder was obtained from Alpha Milk Company (Red Deer, AB, Canada).

2.2.2. Preparation of glycomacropeptide (GMP) from sodium caseinate

Sodium caseinate powder was dissolved in 15 L of autoclaved deionized water (3% w/v) and the pH adjusted to 6.7. The solution was heated to 37°C in a jacketed vessel (Dover Corporation, ELK Grone Village, IL, U.S.A.) supplied with live steam. 90 ml of crystalline rennin at a concentration of 1mg/ml was added to the solution and the temperature maintained at 37°C during hydrolysis. After 30 minutes of incubation, the solution was heated to 75°C for 30 minutes to inactivate the enzyme. The heated solution was cooled to 30°C and the pH of the solution was lowered to 4.6 with 0.2 N HCl to precipitate proteins other than GMP. The GMP and curd mixture were first separated by a stainless steel sieve to remove large curds. A second filtration step with a Buon Vino super jet filter (Buon Vino

Mfg. Inc. Cambridge, ON, Canada), which was equipped with coarse filtration filter pads, was carried out to further clarify the GMP solution. The crude GMP filtrate was separated and concentrated using ultrafiltration modules of different molecular weight cut-offs (MWCO).

2.2.3. Ultrafiltration (UF)

A combination of Millipore tangential flow pellicon cassette UF membranes with 0.46 m^2 surface areas (Bedford, MA, U.S.A.) and Supelco Harp hollow fibre UF membrane cartridges with 0.19 m^2 surface areas (Supelco Inc., Bellefonte, PA, U.S.A.) were used. A Pacific Scientific 1 HP, 3450 rpm pump (Rockford, IL, U.S.A.) and a Motor Master 2000 series control (Minarik electric Company, Glendale, CA, U.S.A.) were used to deliver and control the feed respectively. Three different MWCO UF membrane combinations were used. The first set of trials are depicted in Figure 1, consisted of running the crude GMP filtrate through a Harp 50,000 MWCO UF membrane, collecting the retentate and permeate, followed by concentrating the filtrate with a 10,000 MWCO PLGC pellicon cassette. In the second set of trials (Figure 2), the 50,000 MWCO Harp UF cartridge was replaced by a 30,000 MWCO PTTK Pellicon cassette. Only the 10,000 MWCO UF Pellicon cassette was used in the last set of trials (Figure 3). The retentate and permeate collected in all the trials were freeze-dried before further analysis. The trials were performed in duplicate.

2.2.4. High performance liquid chromatography (HPLC) evaluation

The liquid chromatography system used were made up of the following Shimadzu components: SIL-6A auto injector unit, C-R6A chromatopac integrator, SCL-6A system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada). The procedure of Leonil and Molle (17) was the basis of GMP quantitation. A Pharmacia (Uppsala, Sweden) HR 5/5 mono S column was equilibrated with buffer A (20 mM KCl-HCl buffer, pH 2.0) and elution was obtained using a step gradient: 0-2 minutes, buffer A; 2-12 minutes, a linear gradient of up to 15.3 % buffer B (20 mM KCl-HCl, 1.5 M NaCl, pH 2.0); 12-16 minutes, a linear gradient of 15.3% B to 100% B, holding buffer B at 100% for 5 minutes and reequilibrating the column with buffer A for 13 minutes before the next injection. The run temperature was 40°C at a flow rate of 1 ml/min. The total run time was 35 minutes and uv absorbance was monitored at 214 nm. The peak was collected for amino acid analysis.

2.2.5. Amino acid analysis

The amino acid composition of freeze dried GMP powder and ion-exchange purified GMP was determined with Beckman System 6300 amino acid analyzer, system gold version 6.01. The samples were hydrolyzed in 6M HCl for 24 hours at 110°C before analysis.

2.2.6. Protein determination

The protein content of the freeze dried GMP (retentate) was evaluated with Leco FP-428 nitrogen analyzer (Leco Corporation, St. Joseph, MI, U.S.A) and N=6.38 was the conversion factor used to calculate the protein content.

2.2.7. Yield and purity determination

The GMP yield was expressed as gram of freeze-dried crude GMP powder. The purity of crude GMP was calculated as mg equivalent of sweet whey protein.

2.2.8. Hazard Analysis Critical Control Point (HACCP) Evaluation

The production and separation of GMP were carried out with good manufacturing practice. Hazard analysis critical control point (HACCP) was the key element in product management (18). The initial HACCP attempt was the analysis that looked at what could potentially be a hazard at each stage of the operation. A list of control points (cp) and critical control points (ccp) of the process, where significant hazards could occur, was identified (Figure 2.4,2.5). These cps and ccps were controlled and monitored with appropriate process designs and/or tests. Since this was a new process used in the laboratory, a critical

limit for each preventive measure associated with the respective critical control point was not established. However, two HACCP steps were employed throughout the whole process: 1) looking at the process and product from start to finish; and 2) record keeping. These two steps aimed to identify the critical limit for each critical control point.

In the isolation of GMP from resolubilized sodium caseinate, the potential hazard could be of biological, chemical, physical nature or a combination of the aforementioned. The physical and chemical hazards were largely eliminated by the use of laboratory standard equipments and common mild chemicals (2N NaOH and 2 N HCl). The main potential hazard remaining in the process would be biological or more specifically, microbiological in nature.

The sources of microbial hazards could be from the raw material and/or contamination during the hydrolysis and isolation processes. Two microbial analyses, coliform and standard plate count were used throughout the process to monitor the occurrence of gram negative bacteria and to assess the total microbial load. Samples of the substrate (sodium caseinate) were taken throughout the process flow chart (Figure 2.1, 2.2, 2.3) for microbial analysis. The prevention of contamination was done by: 1) sterilization of all autoclavable equipment that came into contact with the material; 2) sterilization of all water used for solubilization of raw material and for chemical preparation; 3) sanitization of all unautoclavable equipments with 200 ppm chlorine. The chlorine residue was rinsed off with sterile

water; 4) pasteurization of the hydrolysate at 75°C for 30 minutes. Pasteurization also had the added purpose of rennin inactivation.

2.2.9 Antimicrobial assays

The effect of crude GMP on microbial growth was examined using a broth method (19). Frozen or freeze-dried strains of bacteria were inoculated into tryptic soy broth (TSB) and incubated for 24 hours at 30°C. To check for purity, a small amount of this 24-hour broth was streaked out onto a tryptic soy agar (TSA) and incubated for 24 hours at 30°C. A colony was subsequently selected from the TSA plate and reinoculated into TSB under the same conditions and subcultured after 24 hours. The cells were harvested by centrifugation at 10,000 rpm, 4°C for 10 minutes to remove spent broth. The cells were washed twice with phosphate buffer saline (PBS, pH 7.4) by two consecutive resuspensions in PBS and centrifugation at 10,000 rpm, 4°C for 10 minutes. Fresh TSB was used to resuspend the washed cells and serial dilution was carried out to give a final cell count of 5×10^3. Three different levels of cell inoculation, i.e., 5×10^3, 1×10^4 and 1×10^5 were used in all the microbial assay trials. The assays were run in triplicate.

The 30,000 MWCO retentate crude GMP was the chosen substrate because it possessed the highest purity (50%). Three different concentrations of crude GMP were tested, i.e., 0.5mg/50µl, 1.0mg/50µl and 1.5mg/50µl. The positive control was sodium caseinate at a concentration of 0.5mg/50µl. In addition, a suitable antibiotic

(polymyxin B or streptomycin B for Bacillus isolates) was used as the negative control. The overall experimental control was phosphate buffer saline which was the diluent for non-microbial substrates. Commercially obtained glycomacropeptide (Sigma) was used as an additional testing substrate in three of the trials. The growth curve study was carried out in a Macintosh computer controlled Thermomax microplate reader (Molecular Devices corporation, Menlo Park, CA) at room temperature. 150µl of the bacteria cell suspension was transferred into the well of the microtiter plate (Corning Glass Works, Corning, New York, U.S.A.). Then 50µl of the test substrate was added to the wells. The microtiter plate was incubated at room temperature. Growth of the inoculum was monitored by Softmax Kinetic Plate Reader for Macintosh Version 2.3 software. Absorbance readings at 650nm and room temperature were taken at half hour interval over 48 hours. The bacteria strains used in the study were *Pseudomonas aerugenosa* 15442, *Eschericia coli* ATCC 25922, *Listeria innocua* 33090, *Stapylococcus aureus* MJ, Bacillus isolate BLDV2, *Lactococcus lactis* 19435 and *Lactococcus lactis* 11454.

2.3. RESULTS AND DISCUSSION

The theoretical molecular weight of GMP is between 7,000 and 8,000

Daltons, however, GMP has the ability to self associate/dissociate under given pH conditions (20). For example above pH 4.5, GMP is found in a self-associated complex that range in molecular weight between 20,000 and 50,000 Daltons (20). Thus the adjustment of pH (pH 4.6) on the sodium caseinate hydrolysate solution served a two-fold purpose: 1) aggregate the GMP to form larger molecular structures allowing for the use of U.F. membranes with larger molecular weight cutoffs thereby increasing operational flux; 2) to precipitate para-κ-casein and other casein components. A number of combinations of UF membranes (combinations #1: 50,000 and 10,000 MWCO, combination #2: 30,000 and 10,000 MWCO; combination #3: 10,000 MWCO) were examined to evaluate efficiency of separation because of the heterogeneity of higher order aggregates based on environmentally influenced self-association (mono, dimer, tetramer, etc.). The environment dependent aggregation tendency is a well-documented phenomenon in other proteins such as β-lactoglobulin (21,22).

The freeze dried retentates from the three UF combination trials were analyzed for their protein and GMP content (Table 2.1). GMP was found in all of the retentate dry matter samples indicating the diversity of molecular weight distribution. Comparing the percent recovery of GMP from the retentate samples it appeared that the majority of the GMP had conformational sizes between 30,000 and 50,000 Daltons at pH 4.6 (i.e., 50.09 % of the protein recovered in the 30,000 MWCO retentate was GMP). In addition, the presence of GMP in 50,000 MWCO

UF retentate (retentate 1) suggested that some GMP aggregates might have a particle weight of >50,000 Daltons. According to Morr et al. (5) the molecular weight of GMP at pH 7.5 as determined by size exclusion HPLC was found to be 33,000 Daltons (5). Kawasaki et al. (20) reported that the molecular weight of GMP was between 20,000 and 50,000 at pH >4.5. The % GMP/dry matter in retentate was low in all three UF trials. However, despite the membrane combinations used, the total GMP component of the fractioned dry matter was consistent (8.1, 8.3 and 8.0 g for U.F. membrane system 1, 2 and 3, respectively). No diafiltration was performed on the GMP preparations. Hence it was not known whether diafiltration would improve the purity of the separation. The recovery of GMP may be effected by parameters such as ionic interactions on ultrafiltration transmission as influenced by environmental conditions such as pH, ionic strength of bulk, and electric potential of filtering material (23). Nau et al. (23) also noted that the charges of both the molecule and the membrane affect transmission of particles especially those of smaller molecular weight.

Amino acid analysis was conducted on the freeze dried retentate 2 crude GMP fraction (50.1 % purity). The crude GMP amino acid profile did not match the theoretical amino acid values and was presumably due to other contaminating proteins/peptides (Table 2.2). However, upon cation exchange chromatography the amino acid profile closely resembled the theoretical values. Of particular importance was the absence of phenylalanine, an amino acid residue that some

individuals are unable to metabolize properly. People with phenylketonuria must have diets which are restricted in terms of phenylalanine content or mental retardation may result.

It is possible to isolate GMP from casein using a combination of enzymatic hydrolysis (renneting) and ultrafiltration. If highly purified products are required for applications such as protein/peptide sources for phenylketonuria diets, further processing is required.

The microbial load of raw material and intermediate products were tabulated in Table 2.3. The sodium caseinate solutions analyzed have zero coliform count in all eight of the trials, indicating that the quality of the raw material was satisfactory. Coliform bacteria were also absent in all the permeate streams as one would expect because of the low membrane molecular weight cutoffs. Only one of the eight trials performed had a positive coliform count. One possible reason for the presence of coliform bacteria was an isolated incidence of post-pasteurization contamination. In general, the absence of coliform bacteria in the intermediate products indicated that the control points were adequate to prevent coliform contamination.

The standard plate count results also indicated that the quality of raw material was high. The hydrolysis of substrate by rennin at 37°C for one half hour did not increase the microbial floral of the raw material. Samples taken after pasteurization showed that the heat treatment eliminated most of the bacteria. This

ensured that the second stage of GMP production had a good starting point.

The second stage of the process involved the use of different ultrafiltration membranes to isolate and concentrate glycomacropeptide. Again, negative coliform results indicated that the process design was adequate to prevent contamination. The standard plate count results, on the other hand, varied with the membrane used. In trial #1 where a 50,000 MWCO UF was used in series with a 10,000 MWCO UF membrane, the microbial load increased gradually. Most of the microbes were concentrated in the 10,000 MWCO UF retentate. In the second set of trials where a 30,000 MWCO UF membrane was coupled with a 10,000 MWCO UF membrane, the microbial load of all the retentate streams (crude GMP) were high. Glycomacropeptide with the lowest microbial load was obtained in trial #3 where only a 10,000 MWCO UF membrane was used. There are a few factors contributing to the high microbial load of the retentate streams: 1) most bacteria were retained on the retentate side of the membranes because of the low MWCO of the membranes; 2) whenever a two-stage ultrafiltration was used, the hydrolysate was processed twice. The heat generated by the process coupled with prolonged agitation, promoted microbial growth; 3) the smaller the filtration surface, the longer the filtration time and hence the agitation and heating. I.e., 50,000 MWCO UF membrane used in the trials was three times smaller than both the 10,000 and 30,000 MWCO UF membranes. As a result, the retentate stream of the 50,000 MWCO UF membrane had higher than expected microbial count. The high

microbial load of the final product could be improved through the use of larger membrane filtration surface, lower ultrafiltration temperature (4°C) and/or repasteurization of the intermediate and final product.

Antimicrobial assay results indicated that the crude GMP obtained from the ultrafiltration runs did not inhibit the growth of the bacteria strains tested (Figure 2.6,2.7,2.8,2.9,2.10,2.11). There was no significant difference in optical density between the phosphate buffer saline blank control and the three different levels of crude GMP tested. The difference in optical density for the sodium caseinate positive control and the crude GMPs used were also similar. Commercially obtained GMP (Sigma) on the other hand, showed some growth inhibitory effect on *Listeria innocua* 33090 when used in high concentration (1mg/100µl).

2.4. CONCLUSION

Sodium caseinate is a potential source of serial hydrolysis raw material for the production of physicochemically active proteins/peptides. Ultrafiltration may serve as the preliminary step in product isolation by concentration and desalination (diafiltration). The presence of ionic interactions on ultrafiltration transmission as influenced by environmental conditions such as pH, ionic strength of bulk, and electric potential of filtering material (22) may affect the purity of the recovered

product. If a quality product with high purity is desired, a second processing step such as cation exchange chromatography or affinity chromatography is required (19).

Preliminary HACCP results indicate that sodium caseinate is a good raw material for GMP production. The sanitation procedures used in this study are adequate in preventing microbial contamination but not maintaining low total microbial load. The total microbial load may be reduced by: 1) the use of a larger membrane filtration surface; 2) conducting ultrafiltration at 4°C rather than at room temperature; 3) repasteurization of the intermediate or final product.

Table 2.1 Protein and glycomacropeptide (GMP) content of freeze-dried retentates obtained by ultrafiltration of sodium caseinate hydrolysate.

Freeze dried UF fractions	Dry matter recovered (g)	% Protein	% GMP	Total GMP isolated (g)
Trial # 1				
Retentate 1	8.69 ± 0.31	54.14 ± 3.41	30.65 ± 0.94	
Retentate 1A	17.01 ± 0.10	76.23 ± 1.44	31.56 ± 1.41	8.1
Trial # 2				
Retentate 2	10.62 ± 0.17	70.11 ± 7.47	50.09 ± 7.67	
Retentate 2A	11.70 ± 0.18	72.77 ± 1.64	24.18 ± 0.15	8.3
Trial # 3				
Retentate 3	22.86 ± 0.12	76.73 ± 3.31	35.12 ± 2.98	8.0

Table 2.2: Amino acid composition of crude glycomacropeptide and purified glycomacropeptide isolated from sodium caseinate hydrolysates

Amino acids	theoretical value(%)	crude GMP (%)	purified GMP (%)	Sigma GMP (%)
aspartic acid	8.5	0.2	8.8	8.8
threonine	18.2	2.7	16.8	19.5
serine	7.8	5.1	8.1	8.8
glutamic acid	19.2	0.2	19.9	20.5
glycine	0.9	2.6	2.6	3.3
alanine	5.3	63.3	9.3	10.6
valine	8.9	1.3	9.5	1.0
methionine	2	0.2	0.1	0.3
isoleucine	10.1	5.6	10.1	12.3
leucine	1.7	4.0	2.7	2.1
tyrosine	0	0.8	0	0
phenylalanine	0	1.9	0	0
histidine	0	1.2	0.3	0
lysine	5.7	5.5	6.5	6.5
arginine	0	0.7	0	0
proline	11.6	4.6	5.4	6.3

Table 2.3: Microbial load of raw material, intermediate products and by-products of glycomacropeptide production from sodium caseinate solution

Samples	Average Coliform count (CFU/ml)	Average standard plate count (CFU/ml)
Sodium caseinate solution	<1	4.01×10^2
Sodium caseinate hydrolysate solution	<1	3.17×10^2
Pasteurized Sodium caseinate hydrolysate solution	<1	2×10^0
Sodium caseinate curd	<1	1.76×10^3
Sodium caseinate hydrolyzate filtrate	<1	4.00×10^0
30,000 UF retentate	<1	8.40×10^1
30,000 UF permeate	<1	1.44×10^2
10,000 UF retentate	<1	4.85×10^2
10,000 UF permeate	<1	2.10×10^1
50,000 UF retentate	<1	2.68×10^2
50,000 UF permeate	<1	2.30×10^1
10,000 UF retentate	<1	4.61×10^2
10,000 UF permeate	<1	1.00×10^0
10,000 UF retentate	<1	1.48×10^2
10,000 UF permeate	<1	8.00×10^0

Figure 2.1: Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 1).

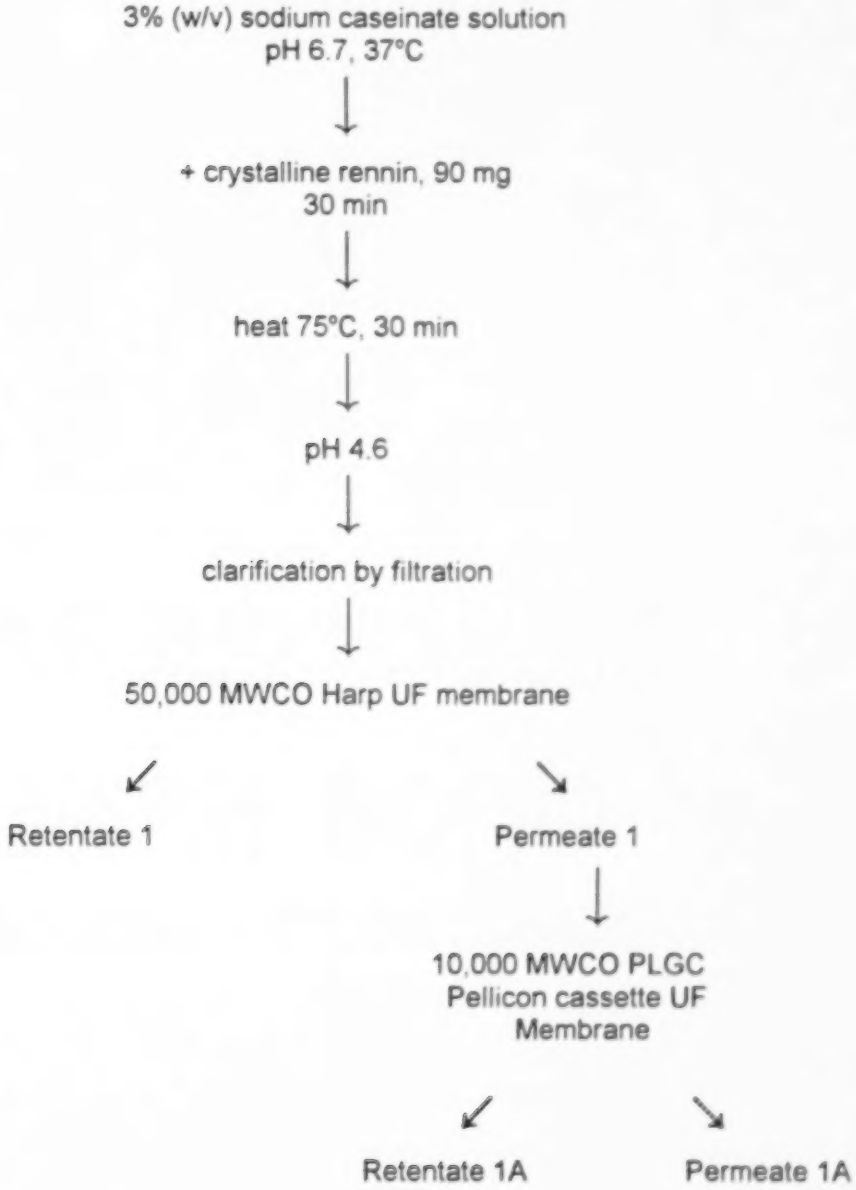


Figure 2.2: Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 2).

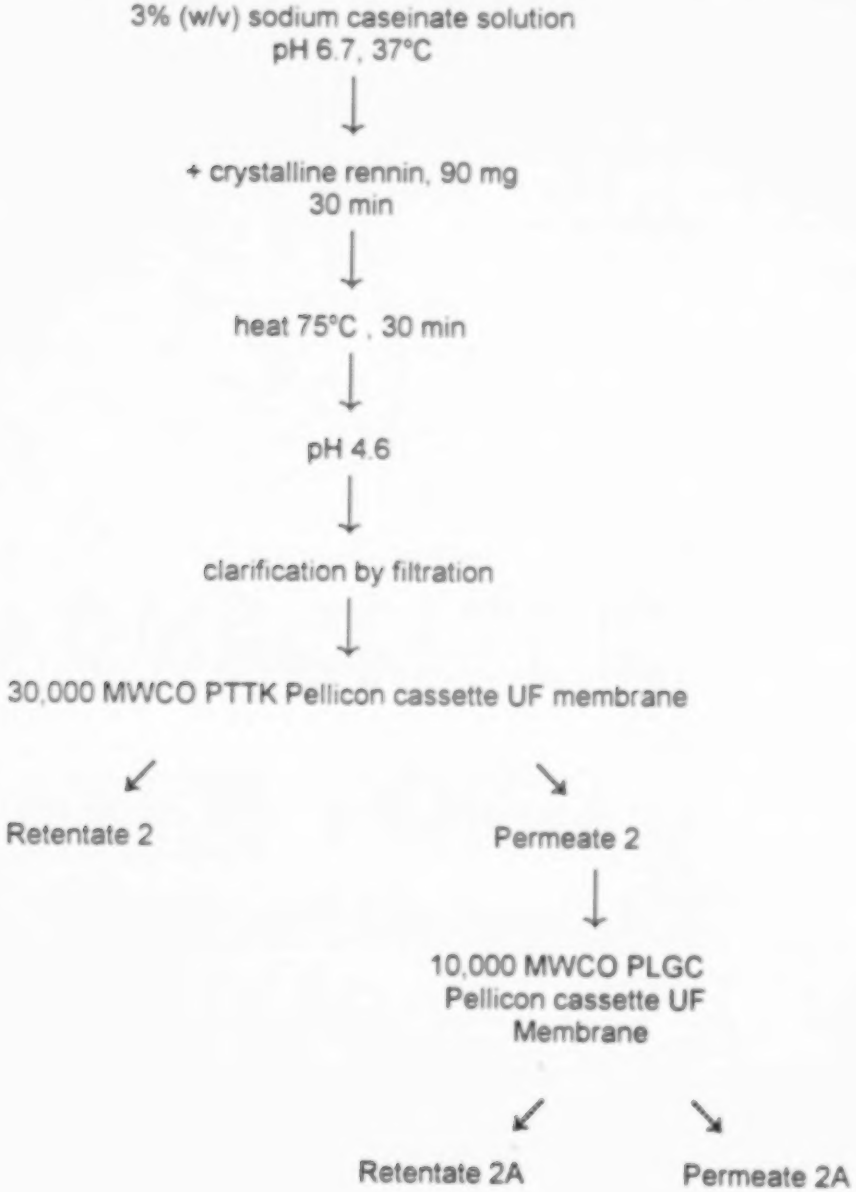


Figure 2.3: Flow diagram of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration (Trial # 3).

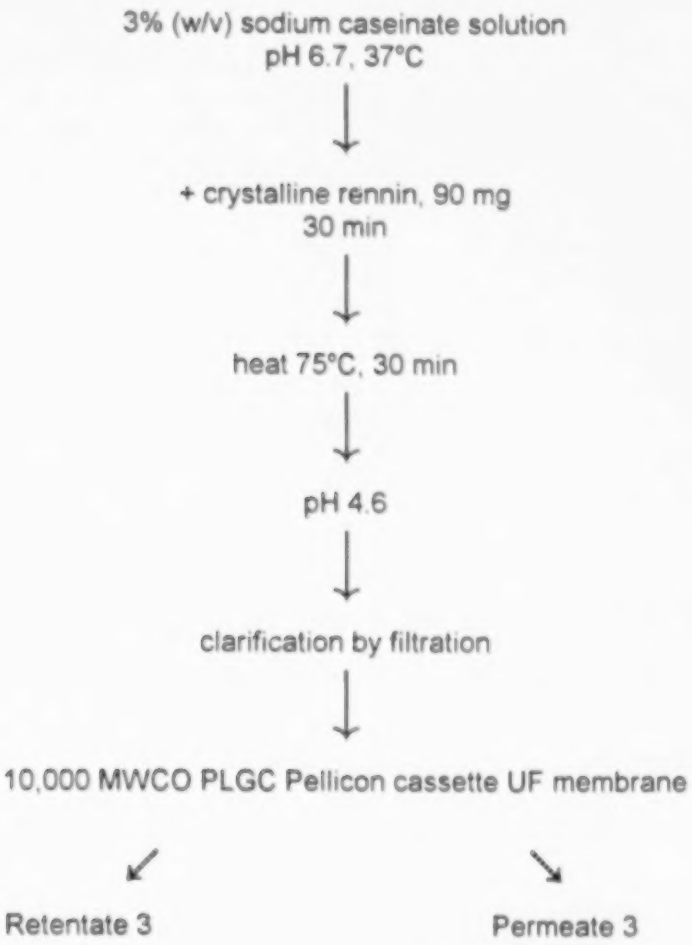


Figure 2.4: Critical points of sodium caseinate hydrolysis

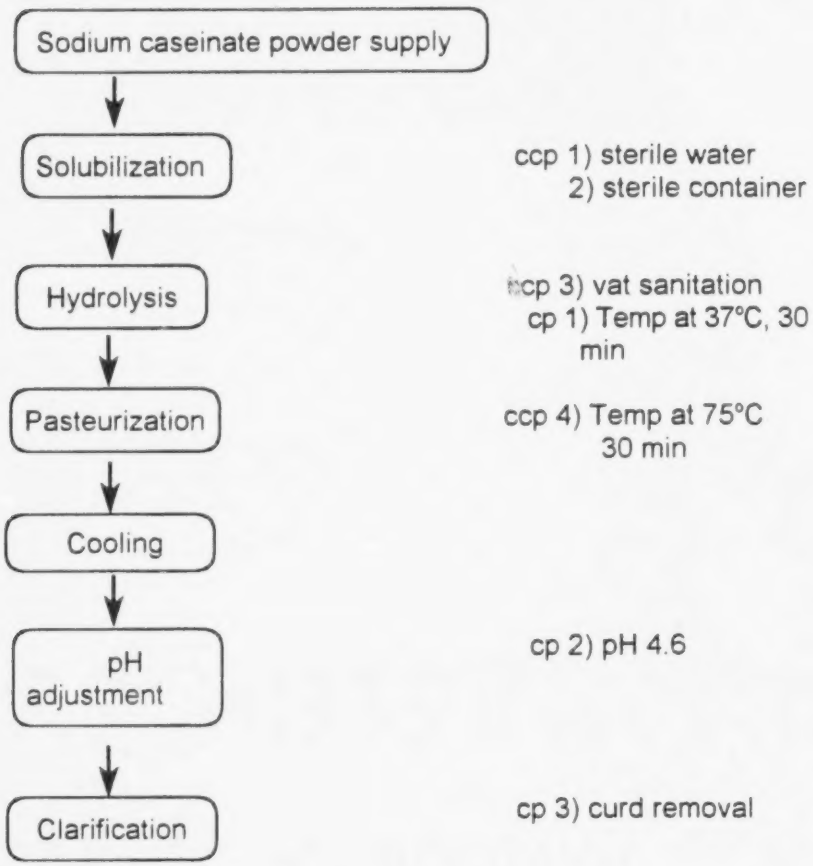
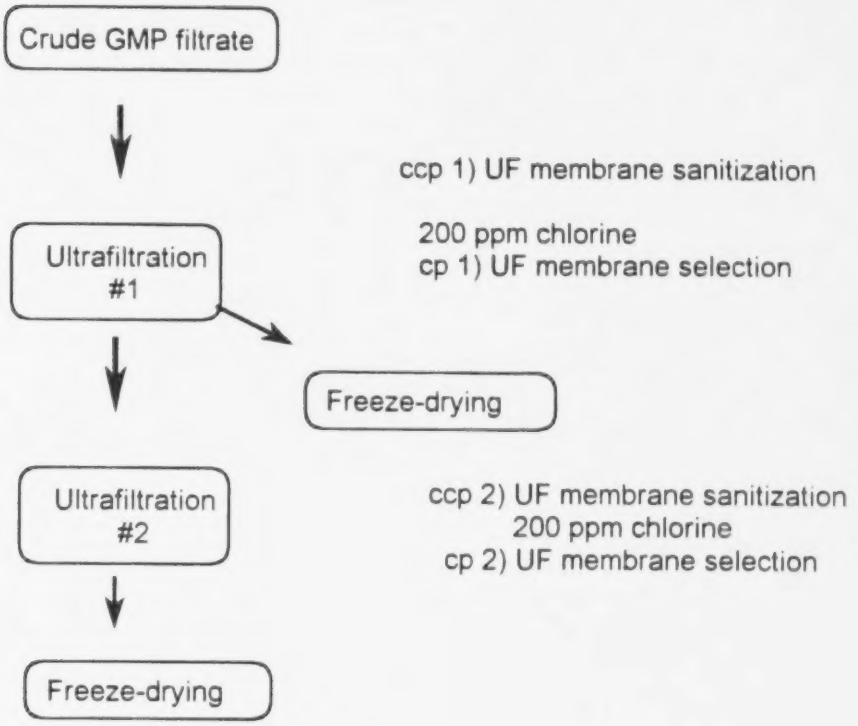


Figure 2.5: Critical points of glycomacropeptide isolation from sodium caseinate hydrolysate by ultrafiltration



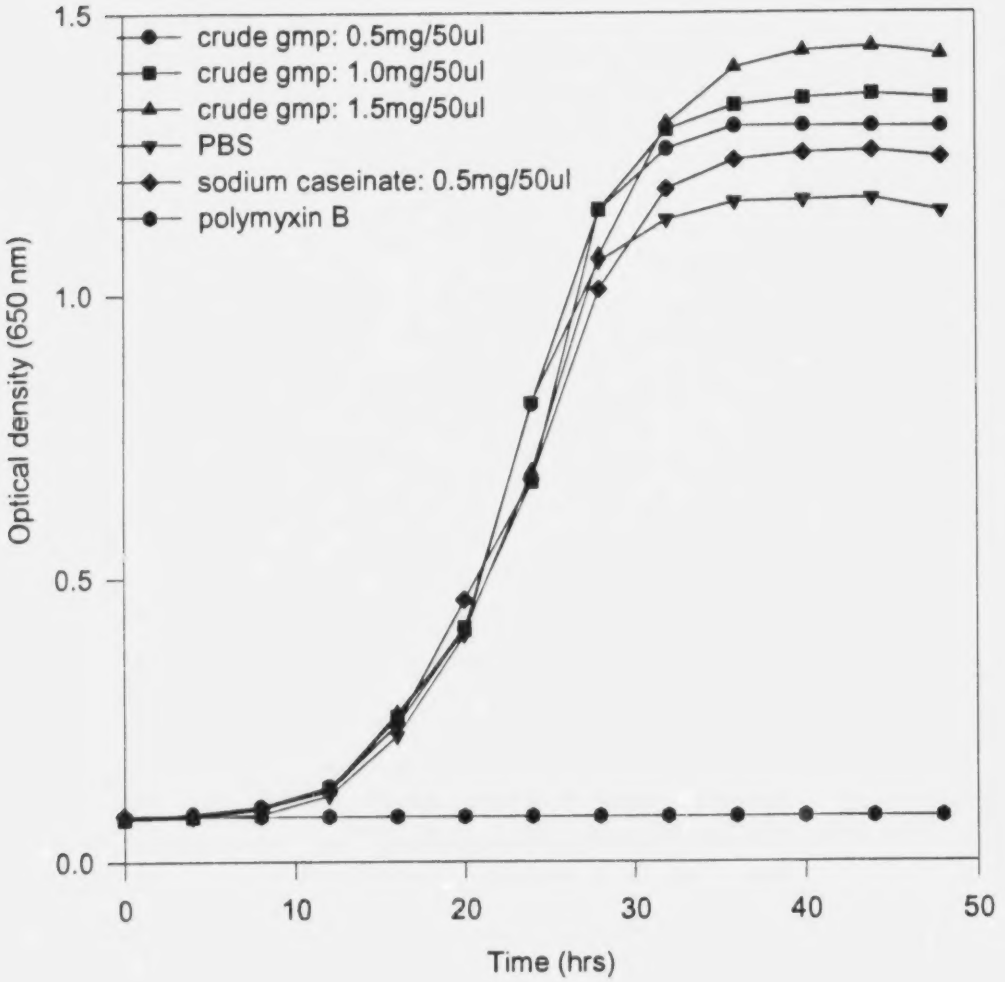


Figure 2.6: Growth of *Pseudomonas aerugenosa* 15442 at room temperature and tryptic soy broth supplemented with various substrates

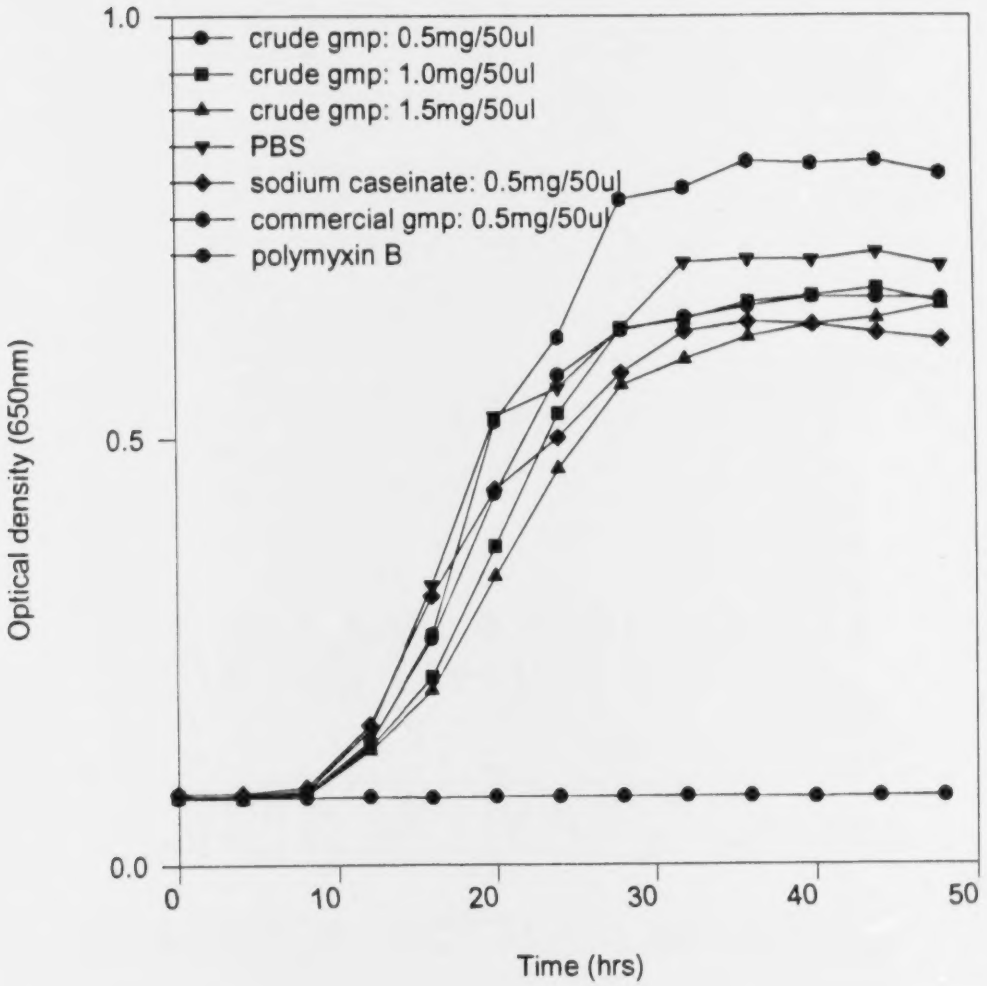


Figure 2.7: Growth of *Eschericia Coli ATCC* 25922 in room temperature and tryptic soy broth supplemented with various substrates

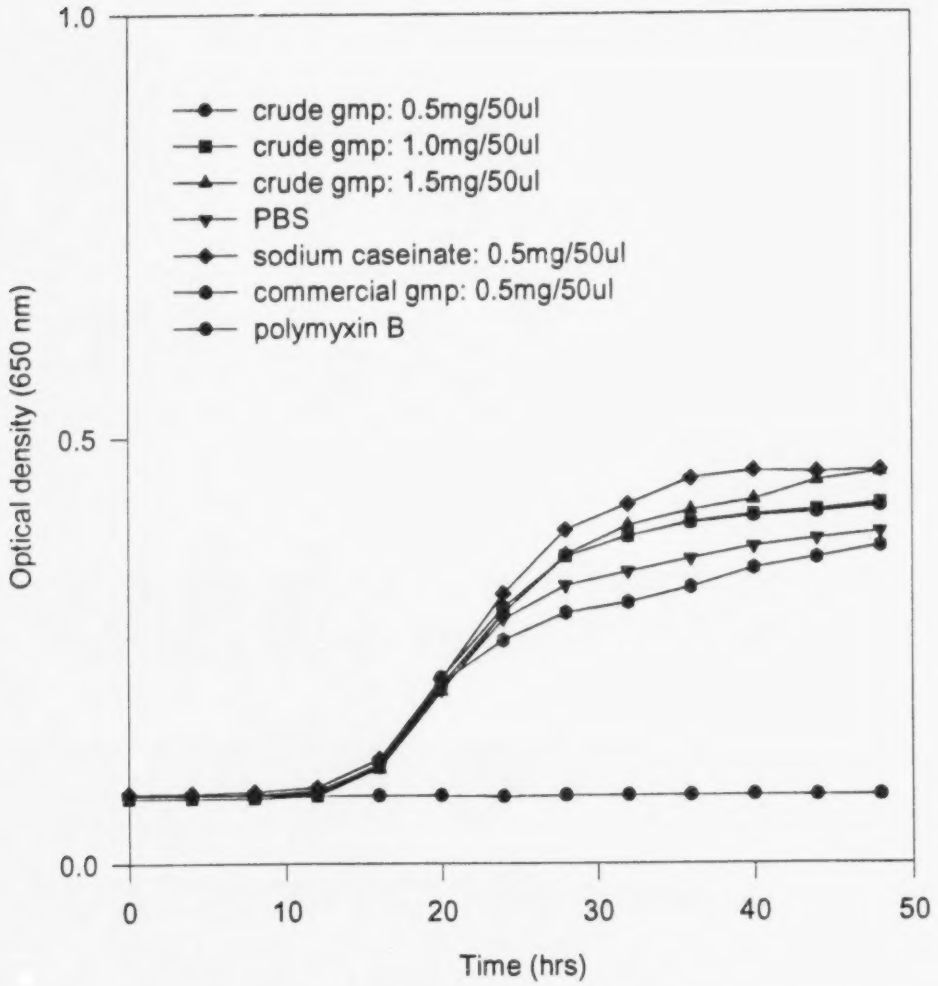


Figure 2.8: Growth of *Listeria Innocua 33090* at room temperature and tryptic soy broth supplemented with various substrates

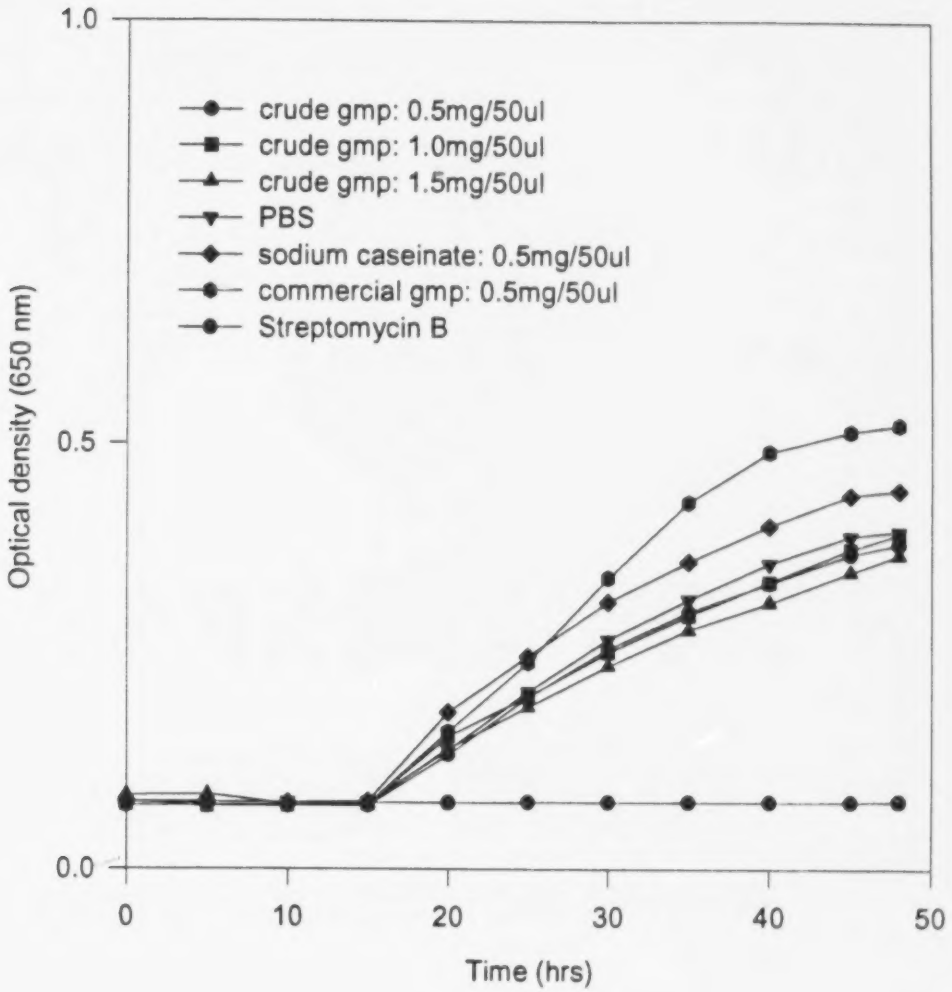


Figure 2.9: Growth of *Staphylococcus aureus* MJ at room temperature and tryptic soy broth supplemented with various substrates

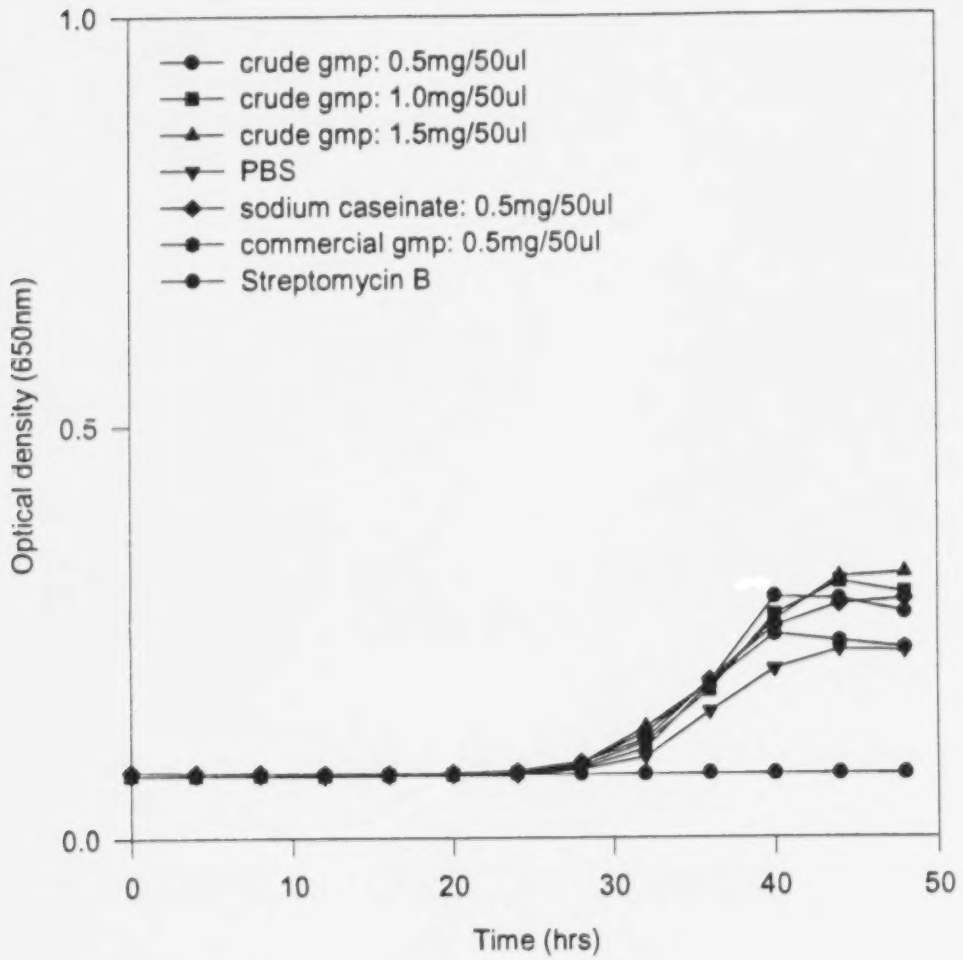


Figure 2.10: Growth of Bacillus isolate BLDV2 at room temperature and tryptic soy broth supplemented with various substrates

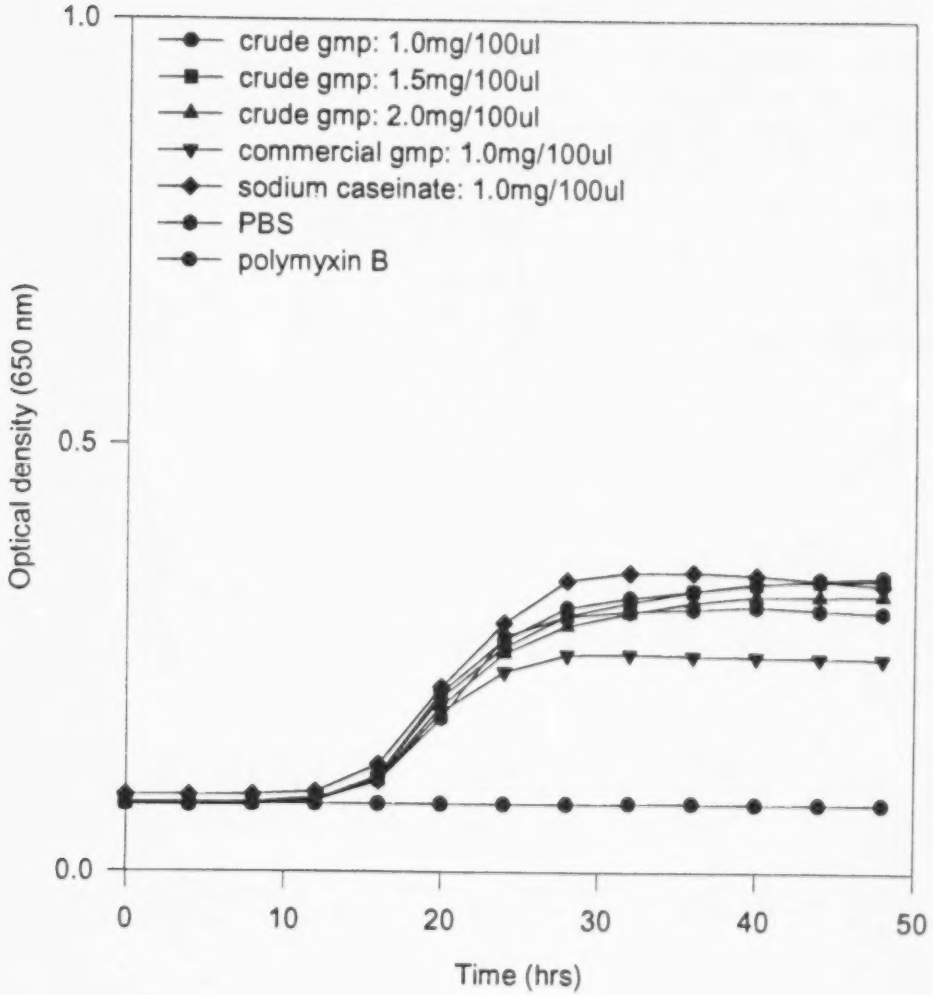


Figure 2.11: Growth of *Listeria Innocua* 33090 at room temperature and trypric soy broth supplemented with 100ul of various substrates

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CHAPTER 3

Effect of charcoal delipidization treatment of β-lactoglobulin on kinetics of β-lactoglobulin/retinoic acid complex and its tryptic hydrolysis

3.1. INTRODUCTION

The binding of β-lactoglobulin to retinoids and fatty acids has been examined extensively (1,2,3,4,5,6,7,8,9,10,11,12). Protein ligand interactions can improve the physicochemical activity of the ligand through increased availability, decreased oxidative degradation and a reduction in cytotoxicity (8,10,11). According to Puyol

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et al. (6) there is approximately one mole of fatty acid associated with one mole of native dimeric β-lactoglobulin. The fatty acids were thought to compete with retinol for binding sites and it was proposed that retinol binding was dependent on the fatty acid concentration (6). In addition, factors such as temperature, pH, ionic strength and dielectric constant that affect the conformational structure of the protein have been shown to affect its ligand binding ability (5,6,8).

Enzymes such as pepsin and trypsin which are part of normal mammalian digestion processes change protein structure through hydrolysis. The effectiveness of the enzymes may be diminished if bound ligands impede the access of the enzymes to the hydrolytic sites on the protein. Variables such as: 1) hydrolysis; 2) protein/retinoid interaction; 3) protein/fatty acid interactions all relate and may be used to help define the bioavailability and physicochemical profile of β-lactoglobulin.

The primary objective of the present work was to evaluate β-lactoglobulin/ligand interaction and hydrolytic patterns with respect to charcoal delipidization treatment.

3.2. MATERIALS and METHODS

3.2.1. Chemicals

β-lactoglobulin genetic variant A and retinoic acid (all trans) were crystallized

products from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lyophilized, dialyzed, crystallized powder of bovine pancreas trypsin and Type II-S soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Activated Carbon powder (Darco G-60) was a product of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

3.2.2. Charcoal treatment

Bound lipids were removed from the β-lactoglobulin variant A using the method of Chen (14). Darco G-60 activated carbon was first prepared by washing with milliQ water then drying at room temperature. 1.5 g of β-lactoglobulin variant A was dissolved in 15.0 ml of milliQ water and then combined with 0.75 g of washed carbon. The pH of the protein/carbon mixture was adjusted to pH 3.0 with 0.2 N HCl before placing in an ice water bath (continuous stirring) for 1 hr. Centrifugation was carried out using a Beckman JA-20 fixed angle rotor at 20,400 x g (13,000 rpm) for 20 min at 2^{0}C. The supernatant was filtered through a 0.22 μm cellulose acetate millipore filter prior to adjusting the pH to 7.0 with 0.2 N sodium hydroxide. The resulting solution was freeze dried.

3.2.3. Fluorescence spectroscopy

The fluorescence measurements were carried out with a Perkin Elmer Luminescence Spectrometer LS 50B using an excitation wavelength of 296 nm and

an emission wavelength of 332 nm (the slit width for both the excitation and emission wavelengths were set at 4.0 nm).

3.2.3.1. β-lactoglobulin titration

Using the methods of Cogan et al. (5) and Dufour and Haertle (15) the following procedures were used to titrate the β-lactoglobulin and retinoic acid: 2 ml of β-lactoglobulin solution (concentration of 2 μM) was placed in a cuvette, and incremental amounts (4 μL) of ligand (retinoic acid in ethanol, 0.30 mM) injected into the protein solution with a Socorex micropipette. The protein/ligand solution was mixed and allowed to stand for 5 min prior to fluorescence measurement. For each titration point, a buffer/ligand blank was prepared and treated in the same manner as the sample. All titration points were run in triplicate.

3.2.4. Determination of the apparent dissociation constants

The dissociation constants (Kd) and apparent molar ratio of ligand/protein (n) at saturation were examined using the direct linear plot method (19). The direct linear plotting method requires that fluorescence (corrected for blank) is plotted directly against the ligand concentration. Using this method, no mathematical manipulation of the data is required as the Kd is obtained directly from the intersection points on the plot.

3.2.5. Enzymatic digestion

The effect of enzymatic digestion on β-lactoglobulin and the β-lactoglobulin/retinoic acid complex was investigated.

3.2.5.1. Trypsin hydrolysis.

The hydrolysis buffer outlined in the Worthington manual (17) (0.05 M Tris-HCl, pH 8.1 with 0.00115 M $CaCl_2$) was utilized as the base solution. To the buffer solution 10 mg/ml of β-lactoglobulin was added along with enough trypsin to accomplish a 1:500 protein/enzyme ratio. The digestion was carried out in a New Brunswick Scientific Controlled Environment Incubator Shaker for 4 hrs at a temperature of 37^{0}C with sampling occurring at 30 min intervals. The enzymatic reaction was stopped in each sample using trypsin inhibitor. Tryptic hydrolysis was carried out for the following protein and protein/ligand combinations: 1) native β-lactoglobulin variant A; 2) charcoal treated β-lactoglobulin variant A; 3) native β-lactoglobulin variant A bound to retinoic acid and 4) charcoal treated β-lactoglobulin variant A bound to retinoic acid. All runs were performed in triplicate.

3.2.5.2. High Performance Liquid Chromatography (HPLC) hydrolysis evaluation of protein hydrolysate.

The liquid chromatography system used consisted of the following Shimadzu

components: SIL-6A auto injector unit, C-R6A Chromatopac integrator, SCL-6A system controller, LC-6A binary pumping system, SPD 10A dual channel detector (Fisher Scientific, Edmonton, Alberta, Canada). The procedures outlined by Sitohy et al. (20) were the basis for the evaluation of the protein degradation. A Nucleosil C_{18} column (Phenomenex, Torrance, CA) was equilibrated in solvent A (0.15 M NaCl/HCl, pH 2.5), and the elution was obtained using a linear gradient from solvent A to solvent B (60 % acetonitrile and 40 % solvent A) in 32 min. The run temperature was 40^{0}C at a flow rate of 1 ml/min with an absorbance reading at 214 nm.

3.3. RESULTS AND DISCUSSION

The β-lactoglobulin and retinoic acid (protein/ligand) binding parameters were evaluated using a direct linear graphical method. The dissociation constant (Kd) at saturation for the charcoal delipidized β-lactoglobulin/retinoic acid complex was 2.10 X 10^{-6} M (Figure 3.1). The Kd for native β-lactoglobulin/retinoic acid complex was 2.8 x 10^{-6} M (Figure 3.2). The two dissociation constants were in the same range, therefore, charcoal delipidization was considered to have little effect on the protein ligand binding dynamics. According to Cho et al. (4) the retinoid binding site was in the interior cleft of the β-lactoglobulin rather than the surface pocket, where the fatty acids had been shown to associate with β-lactoglobulin by surface hydrophobicity (18). In addition, electrostatic interaction had been

proposed to be one of the components involved in free fatty acid/β-lactoglobulin binding (12). The similar Kd values for the native and charcoal delipidized β-lactoglobulin supported the conclusion of binding site diversity for retinoids and fatty acids (1,4,18).

The apparent molar ratio of ligand/protein (n) of retinoic acid to both charcoal delipidized and native β-lactoglobulin were 0.92 and 0.99, respectively and were consistent with published data (13,15). The pH during hydrolysis was 8.1 which favoured the dimeric form of β-lactoglobulin and n was, therefore, calculated using the protein as a dimer (3,11,15,19). Dufour and Haertle (15) examined the apparent molar binding ratios of native and chemically altered (esterification and alkylation by capping the carboxyl group and lysine residue respectively) β-lactoglobulin. The chemical treatments increased the surface hydrophobicity of the protein and changes in n only occurred when the secondary structure of the β-lactoglobulin was opened up which was thought to present a second binding site (15). Dilipidization would have changed the surface hydrophobicity of the β-lactoglobulin, however, no changes in the structure should have occurred which may explain why there were no changes in n after charcoal treatment.

The resistance of β-lactoglobulin to peptic digestion has been reported (19). Like pepsin, trypsin is a digestive enzyme. Trypsin is very specific as it catalyzes the hydrolysis of only those peptide bonds in which the carboxyl group is contributed by either a lysine or arginine residue.

Tryptic hydrolysis of β-lactoglobulin which was 1) native, 2) native bound to retinoic acid, 3) charcoal treated, 4) charcoal treated bound to retinoic acid was conducted (Figures 3.3 and 3. 4). MacLeod et al. (19) reported greater than 90% tryptic degradation of native and charcoal delipidized β-lactoglobulin, however, only one measurement was given which was taken after 4 hrs of hydrolysis. Examination of Figures 3 and 4 indicated that only 30 minutes was required to digest the native and charcoal treated β-lactoglobulin to 90%. While the final digestion percentages agreed with MacLeod et al. (19) for the charcoal treated and untreated β-lactoglobulin (~20%) it was very time dependent. The presence of retinoic acid, presumably in the interior β-barrel where the lysine residues were thought to be involved in ligand binding, significantly reduced the enzyme activity. Protection of the protein might be due to physical blocking of the hydrolytic sites (lysine and arginine) by retinoic acid.

Hydrolysis patterns for the charcoal treated β-lactoglobulin which was unbound and bound to retinoic acid were very similar to the untreated protein (>90% and <24% hydrolysis, respectively). Charcoal delipidization might change the surface hydrophobicity but similar to the molar binding ratios there was very little effect on the tryptic digestion of β-lactoglobulin. β-lactoglobulin is classified as a molecular transport carrier and its' resistance to digestion when bound to retinoic acid may help explain the physicochemical role of the protein.

3.4. CONCLUSIONS

The binding of retinoic acid to β-lactoglobulin that was native and charcoal delipidized as well as the digestive patterns of the aforementioned were the focus of this investigation. In terms of the dissociation constants (Kd) and the apparent molar binding ratio (n) the native and charcoal treated protein behaved in a similar fashion (Kd values 2.1 X 10^{-6} M and 2.8 X 10^{-6} M, respectively and n values of 0.92 and 0.99, respectively). Charcoal treatment changes the surface hydrophobicity of the β-lactoglobulin but does not appear to change either the binding ratio or the dissociation. Changes in hydrophobicity that also cause changes in the secondary structure do impact the binding profile of β-lactoglobulin to fat soluble macromolecules which is thought to be due to the creation of additional binding sites (15).

Mammalian digestion which is aided by site specific enzymes such as trypsin (e.g. arginine and lysine) does appear to be affected by retinoic acid binding but not by charcoal delipidization. There is an ~70% difference in hydrolysis when retinoic acid is bound to either the charcoal treated or native protein. The decreased hydrolysis (20% versus 90% destruction for the unbound β-lactoglobulin) may indicate that β-lactoglobulin may function as a carrier protein for fat soluble macromolecules.

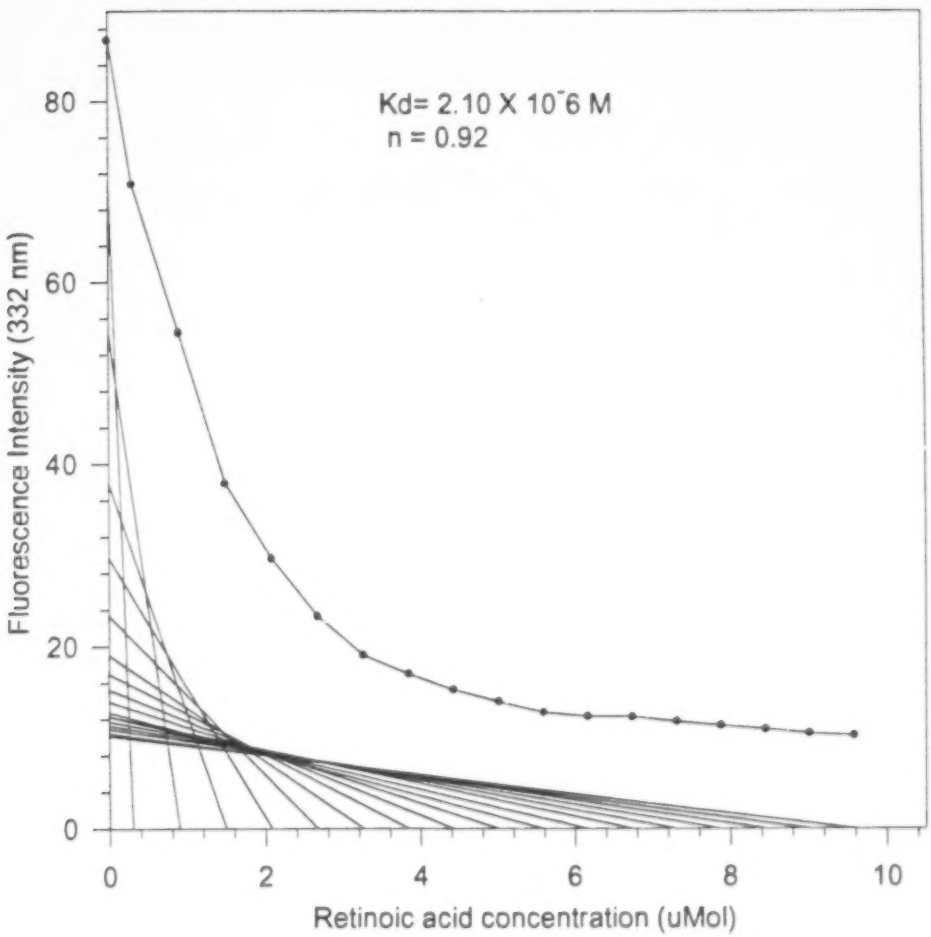

Figure 3.1: Fluorescence titration curve of charcoal treated B-lactoglobulin variant A with retinoic acid and the kinetic parameters (kd and n) of the protein/ligand binding

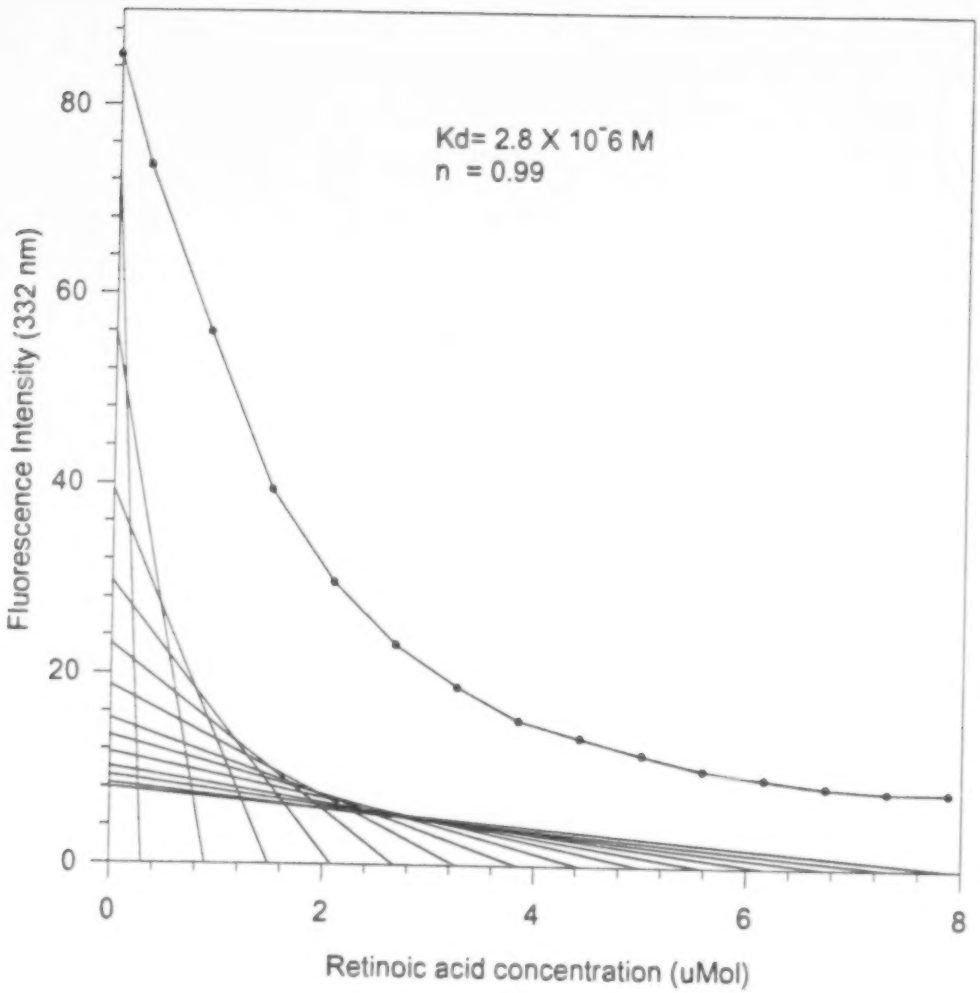

Figure 3.2: Fluorescence titration curve of native B-lactoglobulin variant A with retinoic acid and the kinetic parameters (Kd and n) of the protein/ligand binding

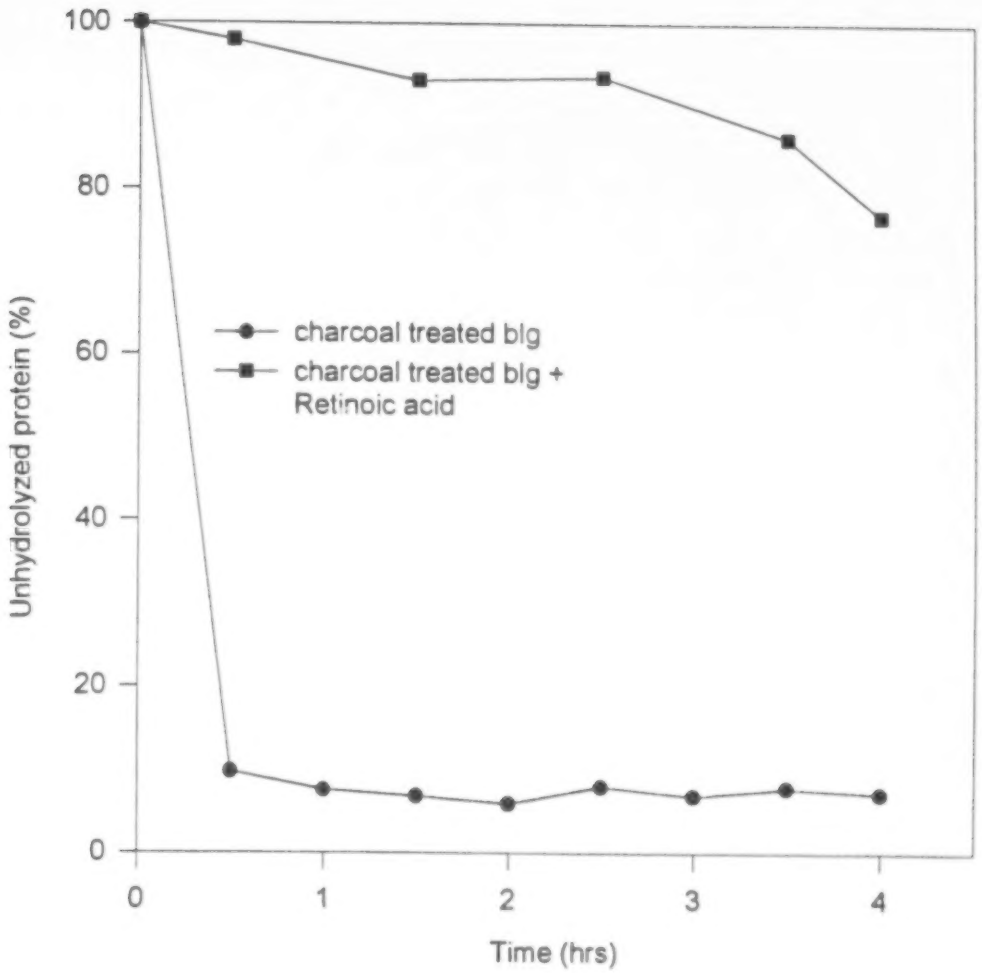

Figure 3.3: Tryptic hydrolysis profile of charcoal treated β-lactoglobulin variant A and charcoal treated β-lactoglobulin variant A/ retinoic acid complex

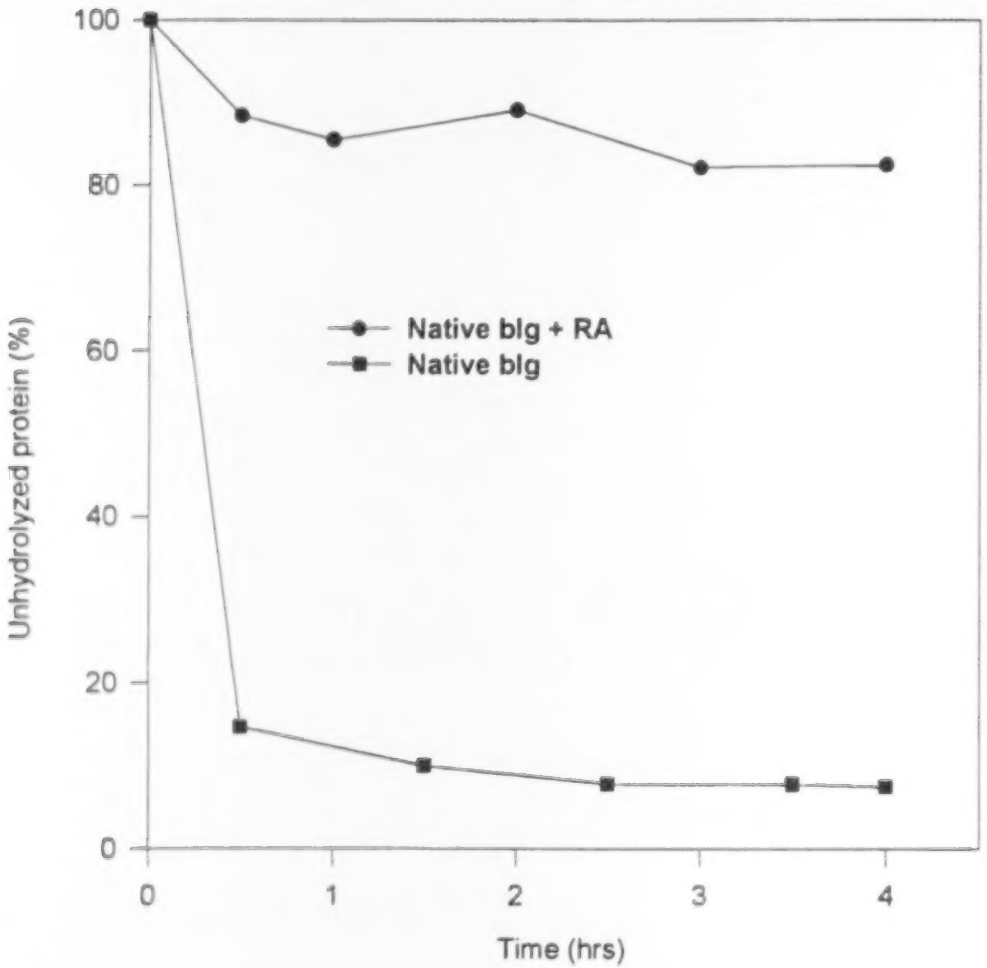

Figure 3.4: Tryptic hydrolysis profile of native β-lactoglobuin variant A and native β-lactoglobulin variant A/retinoic acid complex

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CHAPTER 4

CONCLUDING REMARKS AND RECOMMENDATIONS FOR FUTURE WORK

4.1. SUMMARY OF RESEARCH FINDINGS

Sodium caseinate is a good starting raw material for the production of physicochemically active proteins and by serial hydrolysis, peptides. The application of membrane separation technology may serve as the preliminary step in product isolation, concentration and desalination (diafiltration). Efficiencies of separation and purity of the isolated product by ultrafiltration may be influenced by

environmental conditions such as pH, ionic strength of the medium and electrical potential of the filtering material (1). Membrane processes may be combined with a second processing step such as cation exchange chromatography or affinity chromatography to recover products of high purity (2).

Preliminary HACCP results indicated that sodium caseinate was a good raw material for glycomacropeptide production. The sanitation procedures used in this study were adequate in preventing microbial contamination but not maintaining low total microbial load. The total microbial load could be reduced by: 1) the use of larger membrane filtration surface; 2) conducting ultrafiltration at 4°C rather than at room temperature; 3) re-pasteurization of the intermediate or final product.

Crude glycomacropeptide was not shown to inhibit the growth of *Pseudomonas aerugenosa* 15442, *Eschericia coli* ATCC 25922, *Listeria innocua* 33090, *Staphylococcus aureus* MJ, *Bacillus* isolate BLDV2, *Lactococcus lactis* 19435 and *Lactococcus lactis* 11454. However commercially obtained glycomacropeptide did have have some inhibitory effect on the growth of *Listeria innocua* 33090 as indicated by the lower optical density obtained in the study.

The binding of retinoic acid to native and charcoal delipidized β-lactoglobulin as well as the effect of protein/ligand binding on trypsin digestion were examined in the second investigation. The dissociation constants (Kd) and the apparent molar binding ratio (n) of the native and charcoal treated protein behaved in a similar fashion (Kd values 2.1 X 10^{-6} M and 2.8 X 10^{-6} M and, n values of 0.92 and 0.99, respectively). Although charcoal treatment changes the surface hydrophobicity of

β-lactoglobulin it does not appear to change either the binding ratio or the dissociation. Changes in hydrophobicity that also cause changes in the secondary structure of β-lactoglobulin affects its binding profile to fat soluble macromolecules which is thought to occur by to the creation of additional binding sites (3).

Mammalian digestion of β-lactoglobulin, which is aided by site specific enzymes such as trypsin was affected by retinoic acid binding but not by charcoal delipidization. Digestion of unbound β-lactoglobulin resulted in 90% destruction of the protein. But when retinoic acid was bound to either the charcoal treated or native β-lactoglobulin, only 20% of the protein was hydrolyzed. This decreased hydrolysis indicates that β-lactoglobulin may serve as a carrier protein for fat soluble macromolecules.

4.2. RECOMMENDATIONS FOR FUTURE WORK

There is a tremendous potential for the use of glycomacropeptide and β-lactoglobulin as functional ingredients and food. More work needs to be carried on these two topics so that the biological role of glycomacropeptide and β-lactoglobulin can be fully elucidated. Research on their large scale isolation facilitates future technology transfer to the dairy industry. Improvements in the isolation procedure will also expediate the evaluation of the functional characteristics of glycomacropeptide and β-lactoglobulin. Combining the isolation of

glycomacropeptide from whey with β-lactoglobulin isolation from whey by biospecific subunit exchange affinity chromatography could lead to the 'white mining' of whey which would add value to a traditionally low priced by-product of cheese manufacture.

4.2.1. BIOLOGICAL ROLE OF GLYCOMACROPEPTIDE AND β-LACTOGLOBULIN

The potential of glycomacropeptide as an antimicrobial agent was examined. Results of the antimicrobial assays indicate that further work can be carried out to study the effect of glycomacropeptide on the growth of *Listeria* species. Research in this direction can encompass the following areas: 1) growth curve study on other *Listeria* strains; 2) possible mechanisms involved in the antimicrobial activity of glycomacropeptide. Glycomacropeptide isolated from human milk has been shown to be a Bifudus growth-promoting factor for *Bifidobacterium infantis* S12 at a low concentration of 50 ppm (4). Studies can be carried out to examine the effect of bovine glycomacropeptide on the growth of Bifudus. The potential of glycomacropeptide as a modulator for bacterial clearance from the mouth and as a preventive agent for oral bacteria adhesion to the teeth should be investigated (5).

In terms of its physiological functions, glycomacropeptide has been reported to be effective in reducing gastric secretion and motility in dogs (6,7). Further work

in this regard could reveal a role for glycomacropeptide as a natural dietetic food. The potential of glycomacropeptide as an antithrombotic peptide also needs to be studied further. Glycomacropeptide has been found in the plasma of 5 day old newborn infants after ingestion of cows' milk based formula or human milk (8). However, the role of glycomacropeptide in the blood of newborn has yet to be determined.

In terms of β-lactoglobulin and its ligand binding capabilities, studies should be carried out to examine the ability of β-lactoglobulin to bind other fat soluble macromolecules such as fatty acids and flavour compounds (9). The future research can also examine whether different molecules compete for binding sites on the β-lactoglobulin molecule (10). β-lactoglobulin has been reported to increase the intestinal uptake of retinol in suckling rats (11). The physiological potential of β-lactoglobulin as a retinol carrier can be further investigated by examining the level of retinol in target organs. The effect of heat and chemical treatment on the binding capability of β-lactoglobulin should also be investigated.

4.2.2 ISOLATION PROCESS IMPROVEMENT

Ultrafiltration may serve as the preliminary step in glycomacropeptide isolation by concentration. However, in order to facilitate the study of the biological role of glycomacropeptide, the purity of the final product must be improved.

Improvement in product purity and quality also lends more credibility to glycomacropeptide as a potential commercial source of protein supplement for phenylketonuria diet individuals. The purity of glycomacropeptide isolated by ultrafiltration can be enhanced by coupling ultrafiltration process with diafiltration (12). Diafiltration reestablishes the solutes equilibrium in the solution and thereby improves separation efficiency. A second approach on the improvement of purity is through the use of biospecific subunit exchange affinity chromatography. Glycomacropeptide can be coupled with a solid support matrix to entrap glycomacropeptide ligand. A suitable buffer is then applied to the ligand bound matrix to separate glycomacropeptide. The third method to improve purity is through the use of large scale ion exchange column to specifically extract glycomacropeptide from ultrafiltration retentate (13). All these purification procedures should be carried out at low temperature to prevent microbial growth.

Membrane processes as a separate topic itself also present a challenge in future work. Research conducted to date on glycomacropeptide isolation has concentrated on either ion exchange chromatography or ultrafiltration (12,13). Ultrafiltration process may be modified to accommodate ion exchange or subunit exchange biospecific affinity chromatography to simultaneously concentrate and isolate high purity glycomacropeptide. Sources of glycomacropeptide can also be varied.

4.2.3. SOURCES OF GLYCOMACROPEPTIDE AND OTHER BIOLOGICALLY FUNCTIONAL PEPTIDES

Studies have indicated that glycomacropeptide can be isolated from whey by ultrafiltration or ion exchange chromatography (13,14). The isolation of glycomacropeptide from whey can compliment work conducted in our laboratory on β-lactoglobulin isolation from whey by biospecific subunit exchange affinity chromatography. Research on the isolation of other whey proteins, i.e., α-lactalbumin, lactoferrin, lactoperoxidase and immunoglobulins should be under taken. The 'white mining' of whey will add great value to whey which traditionally is a low price by-product of cheese manufacture.

The isolation of glycomacropeptide from sodium caseinate in this thesis research results in the caseinate curd by-product production. This by-product is a valuable source of biologically functional peptides. Enzymatic hydrolysis of the remaining casein components may yield hypoallergenic peptides (1,15) that can be incorporated into hypoallergenic infant formula.

4.3. REFERENCES

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